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Distribution and diversity of members of the bacterial phylum *Fibrobacteres* in environments where cellulose degradation occurs

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ABSTRACT

The *Fibrobacteres* phylum contains two described species, *Fibrobacter succinogenes* and *Fibrobacter intestinalis*, both of which are prolific degraders of cellulosic plant biomass in the herbivore gut. However, recent 16S rRNA gene sequencing studies have identified novel *Fibrobacteres* in landfill sites, freshwater lakes and the termite hindgut, suggesting that members of the *Fibrobacteres* occupy a broader ecological range than previously appreciated. In this study, the ecology and diversity of *Fibrobacteres* was evaluated in 64 samples from contrasting environments where cellulose degradation occurred. Fibrobacters were detected in 23 of the 64 samples using *Fibrobacter* genus-specific 16S rRNA gene PCR, which provided their first targeted detection in marine and estuarine sediments, cryoconite from Arctic glaciers, as well as a broader range of environmental samples. To determine the phylogenetic diversity of the *Fibrobacteres* phylum, Fibrobacter-specific 16S rRNA gene clone libraries derived from 17 samples were sequenced (384 clones) and compared with all available *Fibrobacteres* sequences in the Ribosomal Database Project repository. Phylogenetic analysis revealed 63 lineages of *Fibrobacteres* (95% OTUs), with many representing as yet unclassified species. Of these, 24 OTUs were exclusively comprised of fibrobacters derived from environmental (non-gut) samples, 17 were exclusive to the mammalian gut, 15 to the termite hindgut, and 7 comprised both environmental and mammalian strains, thus establishing *Fibrobacter* spp. as indigenous members of microbial communities beyond the gut ecosystem. The data highlighted significant taxonomic and ecological diversity within the *Fibrobacteres*, a phylum circumscribed by potent cellulolytic activity, suggesting considerable functional importance in the conversion of lignocellulosic biomass in the biosphere.

Introduction

Cellulose is Earth's most abundant organic polymer and, as such, the microbial-mediated degradation of cellulosic biomass is a fundamental mechanism in the global carbon cycle [29]. Cellulose hydrolysis occurs in both oxic and anoxic environments, where anaerobic decomposition usually occurs due to the synergistic interaction of a consortium of bacteria, rather than the activity of a single species [29]. This is best exemplified in the rumen, where the microbial decomposition of cellulosic plant biomass has been relatively well studied, with members of the genus *Fibrobacter* thought to be the main bacterial degraders of cellulose [8,26,27] in conjunction with *Ruminococcus albus* and *Ruminococcus flavefaciens* [8,39,43,48]. However, *Fibrobacter succinogenes* is considered to be the predominant bacterial degrader of cellulose in the rumen [26], since when *F. succinogenes* strains S85 and A3C were grown in pure culture alongside *R. albus* and *R. flavefaciens*, the *Fibrobacter* species degraded more of the cellulose from intact forage than the *Ruminococcus* species. [7]. This may be explained by the recent observation that *Fibrobacter* species do not appear to utilise either of the two well-established mechanisms of cellulose-decomposition; the aerobic cell-free cellulase mechanism [54], or the cellulosome system typified by anaerobic bacteria and fungi [9]. Instead, the superior efficiency of cellulolysis by *Fibrobacter* species [7] is thought to arise from a novel enzyme mechanism for cellulose decomposition that appears to be restricted to members of the *Fibrobacteres* phylum. The genome of the type strain, *F. succinogenes* S85 does not appear to contain exocellulases or processive endocellulases, and these enzymes are required for both the cellulosomal and free cellulase methods used for cellulose hydrolysis

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[52]. Furthermore, none of the predicted cellulase genes contain the carbohydrate binding molecules, dockerin domains or scaffoldin genes that are typically associated with cellulosomes [46]. Consequently, it has been suggested that the method by which *F. succinogenes* degrades cellulose involves adherence of a putative fibro-slime protein located on the outer membrane of the cell [46] before the severing of individual cellulose chains. These chains are thought to be subsequently transported into the periplasmic space where they are hydrolysed by endoglucanases [53].

F. succinogenes was first isolated from the rumen in 1947 and was originally designated as *Bacteroides succinogenes* [23,24]. However, the subsequent application of 16S rRNA gene-based phylogeny demonstrated that *B. succinogenes* belonged to a separate genus, *Fibrobacter*, that contained two species, the renamed *F. succinogenes* and the newly described *Fibrobacter intestinalis*, both of which were thought to be present only in the mammalian intestinal tract [38].

The diversity of *Fibrobacter* spp. in the herbivore gut has been relatively well characterised, particularly using oligonucleotide probes and comparative sequencing of the 16S rRNA gene. Stahl et al. [45] designed the first *Fibrobacter*-specific oligonucleotide probes. Their three probes had varying levels of specificity, one designed to target all but one of the known *Fibrobacter* strains, one to target rumen isolates, and the other to target caecal strains, enabling the successful detection and quantification of fibrobacters where cultivation-based methods were unsuccessful [45]. Fluorescently labelled oligonucleotide probes were subsequently designed for *F. succinogenes*, *F. intestinalis* and *F. succinogenes* subsp. *succinogenes*, which when used alongside comparative sequencing enabled the characterisation of eight previously uncharacterised *Fibrobacter* strains [1]; five isolated from ovine rumen, two from bovine rumen and one from the bovine caecum [1], with strain identification later confirmed by DNA:DNA hybridisation [2]. Consequently, the application of rRNA-targeted probes enabled the quantification of fibrobacters in the rumen [31,32,45], and the detection of novel *Fibrobacter* populations in the bovine [31,45] caprine [31] and equine [32] intestinal tract. Significantly, Stahl et al. [45] determined that the probe designed to target all but one of the currently isolated, putative *Fibrobacter* strains (then members of *Bacteroides*) detected a greater number of fibrobacters than the combination of rumen- and caecal-specific probes, leading to the suggestion that the bovine rumen contained previously uncharacterised species similar to *F. succinogenes*. This was later supported by the work of Lin et al. [31] which demonstrated that only half of the species detected by general *Fibrobacter* probes in cattle and goats could be detected by probes targeting the two specific species. A further study suggested the presence of novel *Fibrobacter* populations in the equine caecum [32], since application of a *Fibrobacter* genus-specific probe indicated that fibrobacters comprised 12% of the total 16S rRNA in the equine caecum. In addition, while the species-specific probe designed to target *F. succinogenes* suggested that the majority of these sequences belonged to *F. succinogenes*, there was no hybridisation with any of the three *F. succinogenes* subspecies-specific probes, indicating the presence of novel species or subspecies closely related to *F. succinogenes* [32].

F. succinogenes and *F. intestinalis* remain the only two formally described *Fibrobacter* species to date, possibly because fibrobacters are difficult to isolate and cultivate, and their ecology was previously thought to be restricted to the mammalian gut [42]. However, members of a novel subphylum of the *Fibrobacteres*, designated subphylum 2, have since been detected in the gut of wood-feeding termites [19,20] and proteomic analyses has confirmed that these novel *Fibrobacteres* were involved in cellulose hydrolysis in the termite hindgut [51].

Using a genus-specific 16S rRNA gene primer set, members of the genus *Fibrobacter* were detected in landfill sites [35,36] and freshwater lakes [34], providing the first evidence of members of the genus *Fibrobacter* beyond the intestinal tract. These environmental fibrobacters included novel phylogenetic lineages that represented as yet uncultivated species, in addition to *F. succinogenes*-like strains [42]. It has been suggested that fibrobacters are active members of the cellulolytic microbial community in these environments, since it has been demonstrated using quantitative PCR that they become enriched on heavily degraded cotton string both in landfill sites [35] and freshwater lakes [34]. In landfill sites, fibrobacters can comprise up to 40% of the total bacterial rRNA and reach relative rRNA abundances that exceed those detected in the ovine rumen [36].

The molecular detection of novel lineages of the *Fibrobacteres* phylum in landfill sites and freshwater lakes suggests that the true ecology and diversity of this poorly studied, but functionally important phylum, is not fully understood. To address the ecological range and diversity of fibrobacters, we applied *Fibrobacter* genus-specific PCR primer sets to DNA extracted from a range of natural and managed environments where cellulose decomposition occurred, expanding the range of ecological niches for which the presence of fibrobacters has previously been described. Cloning, sequencing and phylogenetic analysis of fibrobacters from seventeen of these environments, in addition to the current diversity of *Fibrobacteres* in the public databases, provides the most comprehensive analysis of the ecology and diversity of the phylum to date.

Materials and methods

Sampling

Sixty-four samples were collected from a range of mammalian gut, terrestrial, aquatic and managed environments, as listed in Table 1. Landfill leachate and water samples were processed by filtration though a 0.2 μm pore diameter membrane. Landfill leachate microcosms were constructed by placing nylon mesh bags containing dewaxed cotton string in 1 L Duran bottles, sterilised by autoclaving and transported to the landfill site where they were filled to the top with leachate in order to avoid the presence of air in the headspace, sealed and incubated in the laboratory at ambient temperature. For solid sample matrices, such as equine faeces, soils and sediments, samples were collected in sterile containers and transported to the laboratory where they were frozen at −80 °C. Samples of cryoconite were collected from three High-Arctic valley glaciers on Svalbard (Austre Brøggerbreen [AB], Midtre Lovénbreen [ML], and Vestre Brøggerbreen [VB]) and three alpine valley glaciers in Austria (Gaisbergferner [GB], Pfaffenferner [PF], and Rotmoosferner [RM]), as detailed by Edwards et al. [12]. In brief, samples were collected aseptically in 15 mL tubes and stored at −20 °C in field stations pending frozen transfer to the Aberystwyth laboratory.

DNA extraction

Either a complete membrane filter (0.2 μm pore diameter) or 0.5 g of sample material was subjected to nucleic acid extraction with phenol-chloroform-isoamyl alcohol and mechanical bead beating using the method of Griffiths et al. [15] with the following modifications. Prior to precipitation with polyethylene glycol, RNase A (Sigma) was added to the aqueous layer at a final concentration of 100 mg mL^{-1} and incubated at 37 °C for 30 min before the addition of an equal volume of chloroform-isoamyl alcohol (24:1) (Sigma), with centrifugation and precipitation as previously described [15]. The DNA was resuspended in 50 μL nuclease-free water (Bioline) and visualised on a 1% agarose (Bioline) gel

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Table 1
PCR and qPCR analysis of environmental samples.

Sample	Sample type	Location	Direct PCR *Fibrobacter* product	Nested PCR *Fibrobacter* product	Clone library sequenced	qPCR (% relative rRNA abundance)[a]
Equine faeces	Faecal matter	n/a	+	+	+	1.31
Ovine rumen fluid	Rumen fluid	n/a	+	+	+	0.04
Bovine rumen fluid	Rumen fluid	n/a	+	+	+	ND
Peat	Peat	Acid erosion complex, Migneint-Arenig-Dduallt, Conwy, Wales	–	–	–	ND
Soil from stable sand dune	Soil	Stable sand dune, Newborough, Anglesey	–	+	–	ND
Blanket bog soil	Soil	Migneint-Arenig-Dduallt, Conwy, Wales	–	+	+	ND
Buckley compost	Compost	Compost heap, Chester, England	–	–	–	ND
Cryoconite VB1	Cryoconite	High Arctic (Svalbard)	–	+	+	ND
Cryoconite PF1	Cryoconite	European Alps (Tyrol)	–	–	–	ND
Cryoconite RM1	Cryoconite	European Alps (Tyrol)	–	–	–	ND
Cryoconite GB1	Cryoconite	European Alps (Tyrol)	–	–	–	ND
Cryoconite ML6	Cryoconite	High Arctic (Svalbard)	–	+	–	ND
Cryoconite AB6	Cryoconite	High Arctic (Svalbard)	–	+	+	ND
Esthwaite (lake) sediment	Sediment	Esthwaite Lake, Lake District, England	–	–	–	ND
Lake Ogwen sediment	Sediment	Lake Ogwen, Gwynedd, Wales	–	+	–	ND
Llyn Aled (lake) sediment	Sediment	Llyn Aled, Conwy, Wales	–	+	+	ND
Aled Isaf (lake) sediment	Sediment	Aled Isaf, Conwy, Wales	–	+	+	ND
Conwy Estuary microcosm 1 cotton	Cotton string	Mussel Bed, Conwy Estuary, Wales	–	–	–	ND
Conwy Estuary microcosm 2 cotton	Cotton string	Mussel Bed, Conwy Estuary, Wales	–	+	+	ND
Conwy Estuary microcosm 3 cotton	Cotton string	Mussel Bed, Conwy Estuary, Wales	–	+	+	ND
Conwy Estuary microcosm 4 cotton	Cotton string	Mud Flat, Conwy Estuary, Wales	–	+	–	ND
Conwy Estuary microcosm 5 cotton	Cotton string	Mud Flat, Conwy Estuary, Wales	–	–	–	ND
Marine off shore transect sediment 1 km	Sediment	Conwy, Wales	–	–	–	ND
Marine off shore transect sediment 2 km	Sediment	Conwy, Wales	–	–	–	ND
Marine off shore transect sediment 4 km	Sediment	Conwy, Wales	–	+	+	ND
Marine off shore transect sediment 8 km	Sediment	Conwy, Wales	–	–	–	ND
Marine off shore transect sediment 12 km	Sediment	Conwy, Wales	–	–	–	ND
Brombrough Dock (landfill) microcosm	Filtered leachate	Brombrough Dock Landfill, Wirral, England	+	+	+	3.90
Buckley (landfill) leachate 1 (LC3)	Filtered leachate	Buckley Landfill, Flintshire, Wales	–	+	+	ND
Buckley (landfill) leachate 2 (LC1B)	Filtered leachate	Buckley Landfill, Flintshire, Wales	–	+	+	ND
Buckley (landfill) leachate 3 (LC2B)	Filtered leachate	Buckley Landfill, Flintshire, Wales	–	–	–	ND
Bidston Moss (landfill) microcosm cotton 1J	Cotton string	Bidston Moss Landfill, Wirral, England	–	+	+	0.02
Bidston Moss (landfill) microcosm cotton 3E	Cotton string	Bidston Moss Landfill, Wirral, England	–	–	–	ND
Bidston Moss (landfill) microcosm cotton 3F	Cotton string	Bidston Moss Landfill, Wirral, England	–	–	–	ND
Bidston Moss (landfill) microcosm 3F containing 0.1% (w/v) avicell	Filtered microcosm	Bidston Moss Landfill, Wirral, England	–	+	+	1.43
Bidston Moss (landfill) leachate 1J	Filtered leachate	Bidston Moss Landfill, Wirral, England	–	–	–	ND
Bidston Moss (landfill) leachate 3E	Filtered leachate	Bidston Moss Landfill, Wirral, England	–	–	–	ND
Bidston Moss (landfill) leachate 3F	Filtered leachate	Bidston Moss Landfill, Wirral, England	–	+	+	ND
Soil transect point 5	Soil[b]	Conwy, Wales	–	+	–	ND
Soil transect point 8	Soil[b]	Conwy, Wales	–	+	–	ND

ND, 'not determined'. Insufficient nucleic acid was retrieved from the environmental sample to enable qPCR with sufficient replication for the quantitative analysis of both general bacteria and *Fibrobacter* spp.
n/a, not applicable.
[a] Percentage relative abundance of 16S rRNA genes of *Fibrobacter* spp. compared with total bacteria.
[b] An additional 24 soil samples from Conwy, North Wales were tested with nested PCR, but no *Fibrobacter* PCR amplicons were detectable.

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Table 2
16S rRNA gene primers used for PCR and qPCR amplification and sequencing.

Primers	Sequence (5′–3′)[a]	Specificity	Annealing temperature (°C)	Amplicon size (bp)	Reference
pA	AGAGTTTGATCCTGGCTCAG	General bacteria	55	~ 1534	[14]
pH′	AAGGAGGTGATCCAGCCGCA				
Fib 1F[e]	CCGKSCCAACGSSCGG	*Fibrobacter* genus	60	~855	[36]
Fib 2AR	ATCTCTCGCYGCGGCGWTYCC				
1369F[b]	CGGTGAATACGTTCYCGG	General bacteria[b]	60[d]	~ 151	[47]
Prok 1492R[b]	GGWTACCTTGTTACGACTT				
FibroQ153F[be]	CCGKSCCAACGSSCGGHTAA	*Fibrobacter*[b] genus	60[d]	~104	[36]
FibroQ238R[b]	CSCCWACTRGYTAATCRGAC				
M13 Forward[c]	GTTTTCCCAGTCACGAC	M13 Vector	n/a	n/a	[37]

n/a, not applicable.
[a] Ambiguities: K = (GorT), S = (GorC), W = (AorT), Y = (CorT), H = (A,CorT), R = (AorG), D = (G,AorT), V = (A,CorG).
[b] Primers used for qPCR analysis.
[c] Primer used for sequencing.
[d] QuantiFastTM SYBR® Green PCR assay (Qiagen) uses the same annealing temperature (60 °C) for all primer sets.
[e] Primers based on those of Lin and Stahl [32] as modified by McDonald et al. [36].

with HyperLadder™ 1 kb (Bioline) before quantification with the Qubit® Fluorometer (Life Technologies) and the Qubit® dsDNA BR Assay Kit (Life Technologies). Cryoconite samples were subjected to PowerSoil® (MoBio Inc.) DNA extraction, as specified by the manufacturer, with DNA being extracted from 250 mg (fresh weight) of cryoconite and eluted in 100 μL Buffer C6. Purified DNA was stored at −80 °C.

Amplification of the 16S rRNA gene with direct and nested PCR

PCR reactions contained 0.2 mM of each primer (Table 2), 0.2 mM of each dNTP, 1× SuperTaq Buffer (Cambio), 0.5 mM $MgCl_2$, 1× BSA, 1 U SuperTaq (Cambio), 50 ng DNA and ddH_2O to a final volume of 50 μL. PCR reactions using the *Fibrobacter* primer set (Fib 1F and Fib 2AR, Table 2) contained an increased concentration of each primer (0.4 mM) and $MgCl_2$ (1.5 mM). PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 1 min at the specific annealing temperature for each primer set (Table 2) and 72 °C for 1.5 min. The final extension was performed at 72 °C for 10 min. For direct PCR, 50 ng of extracted DNA were amplified with the *Fibrobacter*-specific primers (Table 2). Nested PCR consisted of an initial round of PCR using the general bacterial primer set (pA and pH, Table 2), followed by a second round of PCR on the general bacterial amplification products (1 μL) using the *Fibrobacter*-specific primers (Fib 1F and Fib 2AR, Table 2). PCR products were visualised on a 1% agarose (Bioline) gel with HyperLadder™ 1 kb (Bioline) and stored at −20 °C.

Cloning and sequencing of Fibrobacter-specific PCR amplification products

Seventeen of the *Fibrobacter*-specific 16S rRNA gene PCR amplification products from the nested PCR described above were extracted from a 1% agarose (Bioline) gel and purified using the QIAquick® Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. The 16S rRNA gene PCR products were ligated and cloned into competent *Escherichia coli* JM109 (Promega) using the pGEM®-T Easy Vector System I (Promega), according to the manufacturer's protocol. Plasmid DNA was then extracted and purified using the QIAEX® II Gel Extraction Kit (Qiagen) prior to sequencing using the M13 forward primer (Table 2) by Source BioScience.

Quantification of Fibrobacter spp. using qPCR

For each of the five samples for which sufficient DNA template was achieved for qPCR analysis, triplicate qPCR assays were performed with both the general bacterial (1369F and Prok 1492Rb, Table 2) and *Fibrobacter*-specific primer sets (FibroQ153F and FibroQ238R, Table 2) using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Each reaction was performed in a 20 μL final volume, containing 10 ng DNA, 10 μL of 2× QuantiFast SYBR® Green PCR Master Mix (Qiagen), 1 mM (final concentration) forward and reverse primer and ddH_2O. Cycling conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, and 60 °C for 30 s, with fluorescence detection in the combined annealing and extension step. A dissociation step was included at the end of every run in order to confirm the presence of single amplification products.

The amplified 16S rRNA gene of *F. succinogenes* S85 was used to generate standard dilution curves in order to determine the relative abundance of *Fibrobacter* spp. The almost full-length 16S rRNA gene (~1534 bp) was amplified using the primers pA and pH′ (Table 2), as described above, and the amplification product was excised from a 1% agarose (Bioline) gel and purified using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The concentration of purified DNA was established with a Qubit® Fluorometer (Life Technologies) using the Qubit® dsDNA BR Assay Kit (Life Technologies), and the 16S rRNA gene copy number per microlitre was calculated with the following equation: (X g μL^{-1} DNA/[PCR product length in base pairs × 660]) × 6.022 × 1023 = Y molecules μL^{-1}.

Triplicate standard curves of the *F. succinogenes* S85 16S rRNA gene were generated using serial dilutions from 3×10^8 to 3×10^2 gene copies, with all three serial dilutions included on each plate with each primer set. Standard curves for each primer set were generated by plotting the Ct value against the log gene copy number, and a linear line of best fit was used to determine the r^2 value, amplification efficiency and *y*-intercept [41]. The relative abundance (%) of *Fibrobacter* spp. was determined by dividing the number of gene copies per sample from the *Fibrobacter*-specific assay with the number of total bacterial gene copies per sample, as determined by the standard curves for each primer set [44].

Phylogenetic analysis of Fibrobacteres 16S rRNA gene sequences

All sequences classified within the *Fibrobacteres* phylum and annotated as 'good' quality were downloaded from the Ribosomal Database Project [5,6] website in July 2013, and they were combined with sequences derived from the clone libraries produced in this study. The resulting dataset was subsequently aligned using the MUSCLE aligner [10]. Sequences were trimmed to produce an alignment containing only complete sequences corresponding to the regions between positions 188 and 887 of the *E. coli* 16S rRNA gene. Sequences from the aligned dataset were subsequently clustered into operational taxonomic units (OTUs) with a 95% similarity cut-off using CDHIT [21,30]. Sequences were checked for

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chimaeras using Bellerophon [22] and putative chimeric sequences were removed from the dataset. The representative sequences of each OTU ($n = 63$) were aligned using the MUSCLE aligner [10] and imported into ARB [33] where the alignment was visually checked and manually optimised. A maximum likelihood tree was produced from the final alignment using ARB [33]. Nodes for which a bootstrap value of >95% was observed are marked with a filled circle, nodes for which the bootstrap value was between 75% and 95% are marked with an unfilled circle. Nucleotide sequence accession numbers for the representative sequence of each OTU are displayed on each node and the number of sequences clustering within each OTU is displayed in parentheses. Clusters highlighted in grey represent sequences that are affiliated with the two known cultivated species within the genus, *F. succinogenes* and *F. intestinalis*. The scale bar indicates 0.1 base substitutions per nucleotide.

Nucleotide sequence accession numbers

The sequence data have been submitted to the GenBank database under accession numbers KJ364183–KJ364484.

Results and discussion

Genus-specific 16S rRNA gene PCR amplification of Fibrobacter spp. in environmental samples

Fibrobacter spp. were detected using nested PCR in 23 of the 64 samples studied, including equine faeces, ovine and bovine rumen fluids, soils, cryoconite, freshwater, estuarine and marine sediments, and landfill sites (Table 1). This is the first targeted detection of *Fibrobacter* spp. in estuarine sediments, marine sediments and cryoconite. A direct PCR amplification product was detected in 4 of the 64 environmental samples screened (Table 1). This was due to the fact that *Fibrobacter* genomic DNA expresses poor PCR amplification efficiencies [36] and nested PCR greatly improves the sensitivity of detection. Consequently, a direct PCR result for the presence of *Fibrobacter* spp. usually only occurs in samples where there is a significant abundance of *Fibrobacter* spp. The four environments in which *Fibrobacter* spp. were detected with direct PCR (equine faeces, ovine rumen fluid, bovine rumen fluid and Bromborough Dock landfill) are known to have high numbers of fibrobacters, since qPCR has demonstrated that the relative abundance of *Fibrobacter* rRNA compared with total bacterial rRNA was 21–32% (ovine gut) [36] and 28.9% (Brombrough Dock Riser 3) [35].

The relative rRNA abundance of *Fibrobacter* spp. in relation to total bacteria, as determined by qPCR, ranged from 0.02% to 3.9% in landfill sites, which was comparable with previous studies that have shown fibrobacters range from 0.2% to 40% of the total bacterial rRNA molecules in landfill sites [36] and 0.005–1% in lakes [34]. These data suggested that fibrobacters can represent a significant and active proportion of the microbial population in these environments. There are however caveats when using DNA to detect fibrobacters with PCR, as it is thought that DNA-associated molecules interfere with PCR amplification, thus resulting in the previous underestimation of their abundance within the rumen using general bacterial 16S rRNA gene libraries [48]. Furthermore, the extraction method used can also introduce bias [16]. Molecular analysis of reverse transcribed rRNA is thought to be a better approach for studying members of the *Fibrobacteres* [36], as the inhibitory molecules only seem to be associated with DNA. Tajima et al. [48] observed that *F. succinogenes* genomic DNA had a prolonged delay in amplification prior to the exponential amplification phase of the DNA template in qPCR assays; however, once PCR amplification surpassed the threshold of detection, the template amplified exponentially, suggesting that the initial genomic DNA was responsible for the poor amplification efficiency. Thus, when cDNA is used for downstream applications, this potential interference would be overcome. However, it was not possible to extract enough RNA from the samples for RT qPCR to be applied here.

Phylogenetic analysis

The *Fibrobacteres* phylum currently consists of two subphyla, subphylum 2, which only contains species detected in the termite gut, and subphylum 1 (the genus *Fibrobacter sensu stricto*), which contains the two characterised species *F. succinogenes* and *F. intestinalis* (Fig. 1). The generation of new *Fibrobacter* 16S rRNA gene sequence data from several contrasting environments in this study expands our knowledge of the ecological range of this poorly studied phylum, and comparative phylogenetic analyses of these data in addition to the known *Fibrobacteres* diversity in the public databases makes this study of the *Fibrobacteres* phylum the most comprehensive to date. The Ribosomal Database Project repository [5] previously contained only one *Fibrobacter* sequence that had been detected in marine and estuarine sediments, and as a result this study has added to the current understanding of the ecology of the phylum in these and other environments. This is also the first specific detection of *Fibrobacter* spp. in cryoconite, a microbe-mineral aggregate responsible for darkening glacial ice surfaces [49] that is associated with high rates of microbial carbon production despite ambient temperatures between 0 and 1 °C [3].

Previous phylogenetic studies have used 95% similarity to designate species-level diversity within the *Fibrobacteres* phylum due to the substantial 16S rRNA gene and genomic diversity between the two described species [25,42], with the sequences derived from *F. intestinalis* isolates forming a single OTU at 95%, which is below the commonly accepted 97% OTU cut-off used to cluster at the species level [25]. When all sequences were clustered at 95% sequence similarity, 63 OTUs were generated, with *F. succinogenes* comprising 11 OTUs and *F. intestinalis* 3 OTUs that clustered separately from *F. succinogenes* with a bootstrap value of >95% (Fig. 1). This would suggest that the strains currently designated as *F. succinogenes* do not actually represent a single species. Previous studies have suggested that *F. succinogenes* and *F. intestinalis* may actually represent two distinct genera [38,42], but in the absence of phenotypic data to distinguish between the two species (despite significant genomic diversity), elevating each taxon to genus status is premature. Consequently, *F. succinogenes* is currently separated into four sub-species.

In order to determine the extent of 16S rRNA gene diversity within the *F. succinogenes* lineage, all sequences designated as isolates of *F. succinogenes* were downloaded from the Ribosomal Database Project website and aligned as described previously. The alignment was then trimmed to create a near full-length alignment of the 16SrRNA gene (1176 bp) (data not shown). When a similarity matrix was constructed for this alignment, it was found that a 91% clustering value would be needed to group all members of *F. succinogenes* into the same node. Nevertheless, this value for interspecies variation is lower than the current 95% 16S rRNA similarity considered as the minimum allowable within a genus [33]. Clustering at 91% similarity generated 29 OTUs, suggesting that there are at least 27 potentially novel species contained within the phylum, and demonstrating that there is greater diversity outside the two recognised species than within.

The number of sequences contained within each of the 63 OTUs generated at 95% similarity (Fig. 1) varied from 297 sequences to singleton sequences, with 18 OTUs containing only one sequence. As a result, it could not be inferred if these lineages were exclusive to a particular niche, since further sequencing data may well have revealed other as yet undetected fibrobacters that would cluster within these OTUs, either from the same or different environments.

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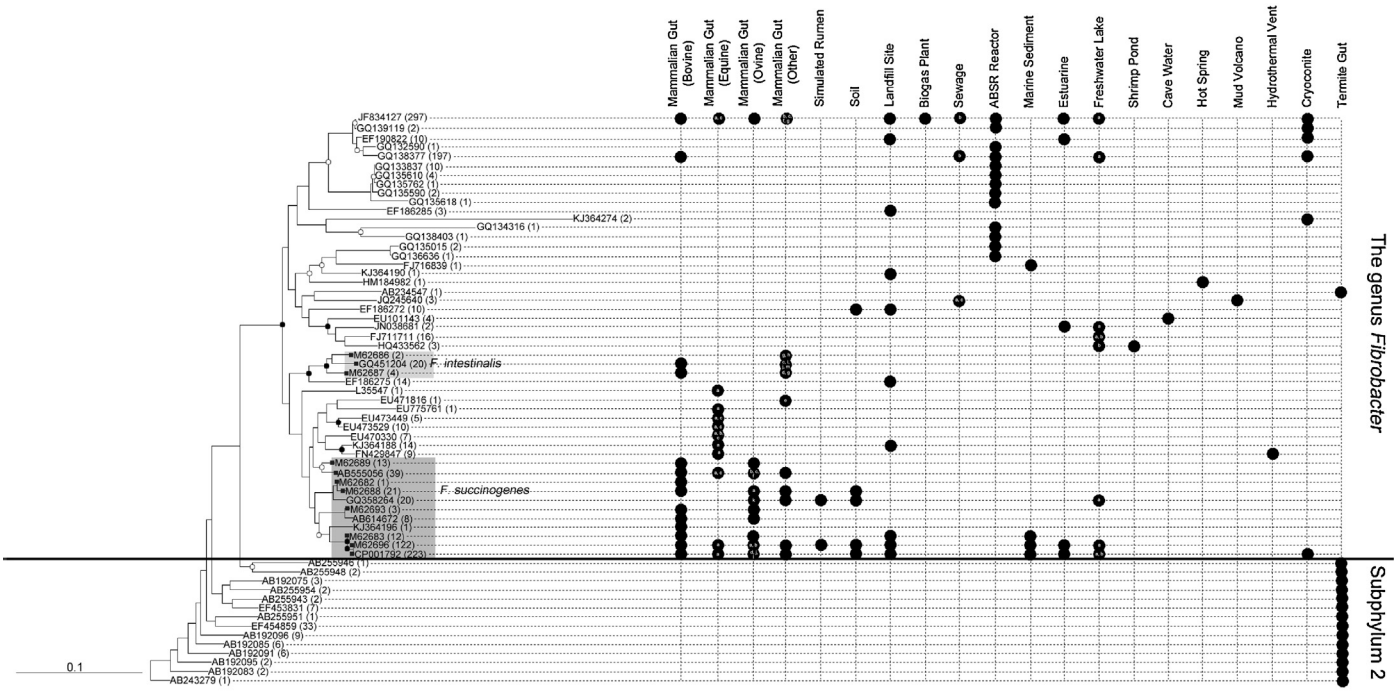

Fig. 1. The ecology and taxonomy of the *Fibrobacteres* phylum. OTUs containing cultivated species are designated by boxes on the end of the node. Refer to Supplementary Table S1 for a full data table describing the sequences contained in each OTU, their accession numbers and percentage environmental composition of each one.
Further information on the environmental distribution is as follows: *Mammalian Gut* (*Equine*): (a) Horse, (b) Grevy's zebra, and (c) Wild ass. *Mammalian Gut* (*Other*): (a) Buffalo, (b) Capybara, (c) Colobus, (d) Dromedary camel, (e) Elephant, (f) Goat, (g) Pig, (h) Rat, (i) River hog, (j) Rock hyrax, (k) Tammar wallaby, (l) Yak, and (m) Yunnan snub-nosed monkey. *Sewage*: (a) activated sludge, (b) anaerobic sludge digester, and (c) raw sewage. *Freshwater lake*: (a) lake sediment and (b) lake water.

Whilst there were lineages that contained species from a range of environments, others seemed to be specific to one particular ecological niche. Seven of the 63 OTUs (at 95%) contained sequences derived from both mammalian gut and environmental samples. However, 24 of the 64 OTUs contained sequences detected exclusively in non-gut environmental samples. These data suggested that a significant proportion of the diversity detected within the *Fibrobacteres* phylum was derived from environmental (non-gut) fibrobacters (Fig. 1). Consequently, the isolation and cultivation of these potentially novel *Fibrobacter* spp. is an obvious priority, in order to further our understanding of their physiology and function in natural and managed environments. The OTU represented by FJ711711 contained species found solely in freshwater lakes, and lineages GQ139119, GQ132590, GQ133837, GQ135610, GQ135762, GQ135590, GQ135618, GQ134316, GQ138403, GQ135015 and GQ136636 all contained species from an anaerobic batch sequencing reactor (ABSR) used for treating swine waste (Fig. 1). In addition, EF186285, EF186275 and KJ364190 contained species found solely in landfill sites, with the separation of EF186275 supported by a bootstrap value of >95%, and KJ364274 contained only species present in cryoconite samples (Fig. 1). Cryoconite fibrobacters were also present in lineage CP001702, which clustered within the *F. succinogenes* group and was supported by a >95% bootstrap value, despite the geographic separation of this environment from grazing areas. In addition, cryoconite bacterial communities have been shown to be distinct from adjacent habitats, and appear to assemble by deterministic processes [13], implying the selection of taxa involved in the functioning of cryoconite ecosystems. Both alpine and Arctic cryoconites receive allochthonous organic matter from plant sources [40,55]. The detection of fibrobacters on all Svalbard glaciers sampled but none of the alpine glaciers examined in this study is interesting. Cryoconite aggregates on Arctic glaciers, including those sampled here, frequently mature to form granular structures [28], while the cryoconite aggregates on the alpine glaciers sampled were poorly developed aggregates of cells, organic matter and mineral debris. The distribution of fibrobacters in Arctic cryoconite is therefore consistent with the evolution of anoxic microhabitats in the interiors of cryoconite granules [18,50]. The presence of both cosmopolitan and unique lineages illustrates the potentially broad dispersal and diversity of fibrobacters in Arctic glacial environments. As such, the data presented suggests a role for fibrobacters in Arctic cryoconite carbon cycling, especially since cryoconite community structure, respiration rates and organic matter profiles are closely related [11,12]. The detection of fibrobacters within this environment is therefore suggestive of a greater role in the global carbon cycle than previously thought. This is supported by the fact that landfill site [35,36], freshwater lake [34] and estuarine fibrobacters were detected on colonised cotton in both this and previous studies.

Nevertheless, the detection of novel *Fibrobacter* species was not limited to environmental samples, as 15 of the detected *Fibrobacter* lineages (95% OTUs) were exclusive to the termite gut (Fig. 1). In addition, the majority of previous mammalian intestinal tract studies have relied on *F. succinogenes* species-specific primers, thus potentially missing other novel members of the genus that may be present in these environments. Bovine, ovine and equine samples were therefore included in order to determine whether or not novel fibrobacters were also present in these environments. Seventeen of the 63 *Fibrobacter* OTUs (at 95%) observed were comprised exclusively of mammalian gut sequences (Fig. 1). Lineages M62682 and KJ364196 (Fig. 1) contained only bovine-associated species, suggesting that there are also as yet unclassified novel species in the bovine rumen. Furthermore, a number of OTUs, EU470330, EU473529, EU473449, EU775761 and L35547, contained only equine-associated species, with the separation of EU473449 and EU473529 supported by bootstrap values of >95%. Lin and Stahl [32] used *Fibrobacter* genus- and species-specific probes in an rRNA hybridisation study of equine caecal contents,

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and the results suggested that the *F. succinogenes* and *F. intestinalis* signal represented only a small proportion of the total *Fibrobacter* abundance generated with the genus-specific probe. These data therefore indicated that novel *Fibrobacter* species were present in the equine caecum, and our detection of five equine-specific *Fibrobacter* lineages supports this assertion.

Further work should focus on the application of PCR-independent methods to investigate the abundance, ecology and physiology of fibrobacters in these environments. The sequence data and phylogenetic analysis presented here now enable the design and application of lineage-specific *Fibrobacter* probes for both RNA and cellular quantification of fibrobacters. Previous studies have also utilised PCR-independent methods, such as RNA hybridisation and fluorescence *in situ* hybridisation (FISH), in order to provide important insights into the abundance, ecology and physiology of *Fibrobacter* lineages in the gut [1,2,31,32,45]. For example, ecological and physiological differences between strains from *F. succinogenes* phylogenetic subgroups 1–3 have been detected in the rumen using qPCR and FISH. Members of *F. succinogenes* subgroup 1 were observed to predominate numerically and were highly active on plant material, particularly on less degradable hay stems, whereas subgroups 2 and 3 were more often associated with other rumen bacteria associated with the more readily degradable leaf sheaths [26,43]. Consequently, such approaches may now be applied to determine the ecology and physiology of fibrobacters in their newly described ecological niches.

Conclusions

There is a current impetus towards better understanding the diversity of cellulolytic microbes and their enzyme systems for biotechnological applications, particularly in the production of second-generation biofuels, and in understanding biomass decomposition and nutrition in commercially important herbivores. Fibrobacters are prolific degraders of cellulose, however, most cultivation-based approaches for the isolation of cellulolytic microorganisms typically focus on aerobic or facultative anaerobic species that are easier to isolate and cultivate, thus disregarding obligate anaerobes such as fibrobacters. Furthermore, the problems associated with *Fibrobacter* DNA amplification have meant that until recently *Fibrobacter* spp. have remained undescribed in many environments due to the apparent biases against the detection of *Fibrobacter* DNA in microbial communities using general 16S rRNA gene and shotgun metagenomic approaches [4,17]. Consequently, the genomic diversity, physiology and metabolism of *Fibrobacteres* members are barely understood, despite the significant ecological, economical and biotechnological potential of this functionally diverse phylum.

In this current study, the understanding of the taxonomic diversity and ecological range of *Fibrobacter* spp. in natural and managed environments has been extended to several newly described niches, all of which potentially promote adaptation and diversity. This has generated novel centres of variation within the *Fibrobacteres* phylum that contain enzymes and growth requirements favourable for biotechnological exploitation. Historically, the ecology of fibrobacters was thought to be restricted to the mammalian intestinal tract. However, the significant diversity of potentially novel *Fibrobacter* species described here and, in particular, the large proportion of OTUs ($n = 24$) derived exclusively from natural and managed environments, demonstrates their broad ecological range in the biosphere. Fibrobacters are therefore an important target for cultivation-based and omics approaches aiming to elucidate novel carbohydrate-active enzymes and mechanisms. It has recently been suggested that *F. succinogenes* S85 utilises a novel mechanism for cellulose hydrolysis [52], and with the observed taxonomic diversity within the *Fibrobacteres* it is likely that the phylum represents a significant source of unexplored diversity with respect to carbohydrate-active enzymes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2014.06.001.

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